**Abstract**

*Jatropha curcas* L., a member of Euphorbiaceae family, is being promoted as a biofuel crop and has attracted great interest in the scientific community. The cultivation in wastelands of *Jatropha curcas* as a biofuel crop avoids the alleged food vs. fuel dilemma. The infestation of collar rot caused by fungus *Macrophomina phaseolina* imposes heavy yield loss in this plant. In this review we highlight the significance of a pathogenesis related gene, JcPR-10a, from this biofuel crop towards stress/defence tolerance. The JcPR-10a recombinant protein exhibit RNase and DNase activity interestingly, the protein also possess antifungal activity against Macrophomina. The docking analysis reveals the binding of three BAP (6-benzylaminopurine) molecules at the active sites of JcPR-10a protein. Furthermore, the overexpression of JcPR-10a gene result in improved shoot regeneration, salinity tolerance and reduced fungal susceptibility in transgenic tobacco. Interestingly, the transgenics also show enhanced endogenous cytokinin level as compared to wild type plants, which, further increased with salinity. Therefore, JcPR-10a gene can serve as an important candidate to engineer stress tolerance in *Jatropha* as well as other plants susceptible to collar rot by *Macrophomina*.

**Keywords**: Cytokinin; *Jatropha curcas*; JcPR-10a; *Macrophomina*; Salinity; Regeneration; Stress Signaling; Transgenics

**Introduction**

The plants evolved from simple algae and later colonized the landmass; the first tiny terrestrial plants to complex seed plants endure environmental challenges for survival. Plants being sessile are strongly affected by climatic changes and pathogenic attack. Furthermore, the plants in nature do not face a single stress at a time but are subjected to different stresses simultaneously. These factors cause metabolic toxicity, membrane disorganization, closure of stomata, decreased photosynthetic activity, generation of reactive oxygen species (ROS) and altered nutrient acquisition [1]. Plants respond and adapt to these conditions during their entire lifecycle with an array of biochemical and physiological changes. A complex network of stress–responsive signal transduction pathways, converge and diverge in co-operation for combating and imparting biotic and abiotic stress tolerance. The molecular and cellular responses to abiotic and biotic stresses include signal perception then signal transduction to cytoplasm and nucleus, gene expression and finally metabolic-biochemical changes leading to stress endurance. Although plants have gradually evolved a remarkable ability to cope with such highly variable environmental onslaughts, the stresses nevertheless represent a primary cause of crop-loss worldwide. The biochemical, physiological or morphological adaptations that allow the growth and survival of plants in response to these stress regimes are important. Deciphering the mechanisms regulating plant's perception to environmental signal and further its transmission to the cellular machinery to activate responses is of critical importance for developing transgenic strategies leading to ameliorate stress tolerance in crops. To meet the increasing demands for plant-based agricultural commodities it would be imperative to enhance productivity of land in current use, expand agriculture to marginal lands and redesigning of crops to cope up with abiotic/biotic stress.

The phytohormones play an important role in stress regulated responses. Different phytohormones, synthesised at low concentrations crosstalk and mediate growth, development, nutrient allocation and source/sink transitions against both biotic and abiotic stresses via synergistic and antagonistic actions [2,3]. Abscisic acid (ABA) is identified as the primary stress hormone, regulating abiotic stresses, however, salicylic acid (SA), jasmonic acid (JA), ethylene (ET), are considered to be the key regulators of plant defence response. Other phytohormones, such as auxins (indole-3-acetic acid [IAA]), cytokinins (CKs), brassinosteroids (BRs), gibberellins (GA) nitric oxides (NO) and strigolactones (SL), either alone or in conjunction with other signaling component or primary stress hormones, participate in plants stress response [4,5].

ABA controls many stress adaptation responses, activation of genes involved in osmotic adjustment, ion compartmentation, regulation of shoot versus root growth and modifications of root hydraulic conductivity [6,7]. There is an overlap in the expression pattern of stress genes under cold, drought, high salt and or ABA application [8]. SA is an inducer of disease resistance by activating systemic acquired resistance (SAR) in plants [9]. The plant defence response is not linear but a complex signaling network regulating crosstalk to networks in other plant functions [10,11]. The plant's innate immune system mainly consists of two interconnected branches termed PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI) [12]. PTI is elicited by pathogen/microbe-associated molecular patterns (PAMPs/ MAMPs) and ETI is triggered by plant disease resistance (R) proteins that activate highly efficient defense reactions upon specific recognition of pathogen effectors. PTI and ETI activate local immune responses and long distance defence reactions, such as SAR [13].

**Keywords**: Cytokinin; *Jatropha curcas*; JcPR-10a; *Macrophomina*; Salinity; Regeneration; Stress Signaling; Transgenics

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Cytokinins are plant hormones, which influence a wide variety of developmental and physiological processes in plants, including cell division, apical dominance, nutrient mobilization, leaf senescence, vascular differentiation, photomorphogenic development, shoot differentiation and anthocyanin production [14], and also integrate different environmental cues [15]. In plants such as Arabidopsis thaliana, the key enzymes involved in CK metabolism are adenosine phosphate-isopentenyl transferases (IPTs) and CK oxidases/dehydrogenases (CKXs) [16,17].

Pathogenesis related (PR) proteins have been defined as proteins encoded by the host plant but induced by various types of pathogens such as viruses, bacteria, and fungi and also by the application of chemicals that mimic the effect of pathogen infection or induce similar stresses. Accumulation of PR proteins is triggered by pathogen attack (biotic), biotic stress, hypersensitive response (HR) and SAR. PR proteins have been classified into 17 different families, ranging from PR-1 to PR-17 based on their primary structure, serological activity, and biological activity in mono and di-cotyledonous plants [18].

The PR-10 is a multigene family differing from other PR proteins in being intracellular, small and acidic with similar 3D structures. Based on similarities in their amino acid sequences, subcellular location and putative function, they are classified into two distinct groups: intracellular pathogenesis-related proteins (IPR) with homology to ribonucleases and (S)-norcoauranine synthases (NCS). The PR-10 IPR group encodes polypeptides of 151-162 amino acids with molecular weight of 15-18 kDa, show cytosolic localisation and conserved three-dimensional structures. PR-10 gene family has been identified in wide variety of plant species and show low- and higher-inter-specific variations [19,20]. The PR-10 proteins are interesting multifunctional proteins playing an important role in plants response to intrinsic and extrinsic factors, however the signaling pathway involved in its activation remains unclear. The major role of PR-10 proteins is reported in response to biotic and abiotic stresses. The induction of PR genes in response to pathogens and parasites ranging from microscopic viruses to insect herbivores and to different environmental cues qualifies their deployment as important gene for multiple stress tolerance. Most PR proteins are induced through the action of the signaling compounds such as SA, JA, ABA or ET, and possess antimicrobial activities in vitro through hydrolytic activities on cell walls, contact toxicity, and hence are involved in multiple stress signalling [21].

The PR-10 protein interacts with stress signalling molecules and cross talk with different stress signal transduction pathways. The RNase activity, ligand binding activity, posttranslational modification (phosphorylation) and phytohormone signaling of PR-10 proteins provide an insight into the mechanism by which these proteins perform their function in the plant system [21]. Studies on upstream region of different PR-10 genes indicate the presence of cis-acting elements for WRKY, RAV1, bZIP, ERF, SEBF and Pit4 transcription factors indicating the role of these transcription factors in regulating PR-10 gene [21]. The regulation of PR-10 proteins also bears relationship in activating other PR-10 proteins, silencing of MtPR10-1 from Medicago truncatula induced other PR proteins and increased tolerance against infection with Aphanomyces euteiches [22]. Some specific function are also observed in PR-10 proteins, Hyp-1 encoding an enzyme for hypericin (HyH) biosynthesis shows 45% homology to Bvt1 class allergens [23]. The AoPR1 gene is involved in phenylpropanoid pathway [24].

Jatropha curcas, a Euphorbiaceae member has been considered as a potential biofuel crop [25,26]. The Jatropha possesses many other good attributes like drought resistance, capability of growing on marginal non-arable lands avoiding food vs fuel dilemma, and has immense potential in reclaiming wastelands [27]. Although, a lot of enthusiasm and excitement exists over the potential of Jatropha, uncertainties too exist, because of which there have been setbacks in efforts towards its domestication [28]. Jatropha shows large variation in seed and oil yield depending upon quality of planting material, soil, climate, agro-practices adopted and different biotic stresses subjected by nature. Among the biotic stresses, many pests and diseases have been reported to be the cause of yield uncertainties. These diseases include collar rot, leafspots, seedling blight, anthracnose, fruit rot, and seedling stem rot caused by fungus, while viral infestation has also been observed. Among the fungal diseases, collar rot caused by Macrophomina phaseolina (Tassi) Gold caused mortality and yield loss at one of our J. curcas plantations in 2009 and recently the same has also been reported by Johnson et al. [29]. Macrophomina is soil borne, anamorphic, ascomycetes fungi with a broad host range infecting over 500 different monocotyledonous and dicotyledonous species [30]. Microsclerotia in soil, seed or host tissue serve as inoculum and plants die/ decay due to production of fungal toxin phaseolinone. The dormant microsclerotia of the fungus can infect a plant host when favourable opportunity for growth occurs and causes many diseases like damping off, seedling blight, collar rot, stem rot, charcoal rot and root rot in various economically important crops [31]. A number of management strategies have been employed to reduce the negative effects of M. phaseolina. Collar rot incidence can also be reduced through proper fertilization, weed control, and irrigation of the crop. Considering the difficulties in managing collar rot disease in J. curcas with traditional methods, and realising that genetic engineering may be the best alternative to find a solution to this disease. Therefore, in this review we highlight the multifarious role of JcPR-10a gene, isolated, cloned and characterized from J. curcas, for enhancing the stress potential of Jatropha.

Structure and transcript regulation of JcPR-10a gene

JcPR-10a cDNA (NCBI accession number JN566052) has an ORF of 483 bp and was isolated from J. curcas growing in the wastelands of India. In the phyogenetic tree the Jc-PR-10a gets clustered with dicots along with Ricinus, Populus and Capsicum PR-10 proteins. The genomic organisation of JcPR-10a consists of two exons and one intron. In Jc-PR-10a the intron is present at 185 bp, similar to PR-10 gene in Vitis vinifera [20]. The signal peptide was not found in JcPR-10a suggesting that it is a cytoplasmic protein like other PR10 IPR proteins. The JcPR-10a has a glycine rich P-loop motif (GNGGGTTK) between amino acid 47-55. This motif is highly conserved with other PR-10 protein as GxGxGxxK, where x represents any amino acid [32]. The three amino acids E97, E150 and Y152 amino acids along with P-loop are supposed to play a role in RNase activity of JcPR-10a protein. Post translational modification especially phosphorylation might be involved in regulating the function of PR-10 genes, as JcPR-10a protein shows the presence of phosphorylation sites, however, further studies need to be carried out. Transcript expression of JcPR-10a was upregulated in response to different stimuli such as NaCl, SA, methyl jasmonate and M. phaseolina. In response to SA and Macrophomina the transcript was found increased at 48 h, however, in case of NaCl and MeJa a strong induction was observed at 12 h, which decreased at 48 h. We first time report the transcript up regulation of JcPR-10a gene by Macrophomina, a pathogen causing collar rot in Jatropha [32].

In vitro characterization of JcPR-10a protein

RNase activity of recombinant JcPR-10a protein: JcPR-10a protein shows RNase activity at a wide pH range (4-9) and at short
time duration, as early as 5 min, indicating that JcPR-10a protein shows strong RNase activity [32]. RNase activity is reported from many PR-10 proteins, and it is considered that ribonucleolytic activity facilitates the plant's defense mechanisms, being released by infected host cells and acting directly on pathogens or during programmed cell death (PCD) at and around the plant infection sites [18]. Zubini et al. [33] has shown a correlation between RNase hydrolysis and cytokinin binding; on incubation of Pru P1 1.01 recombinant proteins with zeatin, the RNase activity was inhibited for 1 h and later restored. This correlation suggested a mechanism of alternate zeatin/RNA binding to protein for its specific functioning, and is an important indication of RNase activity regulating disease resistance via cytokinin signaling.

**Cytokinin binding affinity of JcPR-10a:** The PR-10 proteins show general ligand binding, where both PR-10 and ligand show mutual conformational changes allowing transport of ligands from cytosol to their receptors [34-36]. The binding of PR-10 proteins with different physiological ligands including cytokinin, flavonoids and fattyacids has been reported [37]. The 3D-ligand binding software predicted the presence of cytokinin binding sites in the JcPR-10a protein [38]. Further, the docking studies of JcPR-10a protein explicitly showed that three molecules of BAP bind at its active sites [39]. The putative 3D structure of JcPR-10a protein shows similarity to the other Betv1 protein structures [32]. The Betv1 contain the polyketidecyclase domain involved in binding/transport of lipids and in the synthesis of compounds like pigments, antibiotics and anti-tumour drugs. The polyketidecyclase/dehydrase-like domain of Betv1 is ubiquitous domain, involved in binding of large hydrophobic ligands [40]. An important feature of PR-10 proteins is a large, Y-shaped hydrophobic cavity that could be responsible for the intracellular transport of apolar ligands, as diverse as fatty acids, flavonoids, cytokinins or brassinosteroids. Slight modifications of the structure and shape of this cavity would allow binding of different ligands, that would cause PR-10 proteins to perform diverse roles in plant stress signaling and development [20]. Studies on the ligand binding property of PR-10 are gaining momentum with special emphasis on cytokinins.

**Functional validation of JcPR-10a protein**

**JcPR-10a transgenics exhibit improved shoot regeneration:** The JcPR-10a transformed tobacco leaf explants showed higher number of shoot bud induction and well differentiated shoots on regeneration medium as compared to vector alone on regeneration medium [39]. The higher shoot bud induction could be attributed to the increased cytokinin content in the transgenics and the increased cytokinin content in transgensics could be attributed to the affinity of Jc-PR-10a protein with BAP, which may be the cause of high shoot regeneration in the transgenic lines. The cytokinins play a pivotal role in shoot organogenesis [41]. The cytokinin and auxin hormones in vivo regulate cell division, differentiation and meristem establishment. The Jc-PR10a overexpression lines maintain higher endogenous cytokinin/auxin ratio, therefore the explants showed higher shoot regeneration with no callusing [39]. This phytohormone ratio play an important role in plant tissue culture development, these hormones have antagonistic as well as synergistic roles [42]. The variations in cytokinins to auxin ratios favour development of either shoot or root meristems [43].

**JcPR-10a transgenics exhibit enhanced salinity tolerance:** The tobacco transgenics followed Mendelian segregation ratio of 3:1 Hyg+/Hg' and showed the single copy gene insertion. The morphological, physiological, biochemical and cytokinin levels, which serve as an indicator of salinity tolerance suggested that transgenics were better adapted to salinity stress. JcPR-10a transgenics showed enhanced salt tolerance, as was evident by increased germination rate, shoot and root length, relative water content, proline, soluble sugar and amino acid content under salinity. The enhanced tolerance of the JcPR-10a transgenic could be attributed to increased RWC, higher MSI, reduced electrolyte leakage, ionic accumulation and oxidative damage. Furthermore, the higher level of endogenous cytokinin in JcPR-10a transgenics, which is further increased on exposure to salt stress and might be involved in mitigating the salinity-induced damage. The increased cytokinin levels can enhance the resistance against salinity by functioning as antioxidants [44]. Increased cytokinin levels maintain high cellular redox potential during drought and hence reduce damage by reactive oxygen species (ROS) [45]. Zwack and Rashotte [3], mentioned the complex role of cytokinin in abiotic stress responses, suggesting that the cytokinin concentrations show a transient increase on initial stress, followed by maintenance of increased cytokinin concentrations with increased stress conditions. Similarly, the JcPR-10a transgenics showed an increased level of endogenous cytokinin during initial low salinity stress as compared to WT and further maintained almost similar concentration of cytokinins at higher salinity stress in transgenic plants. Interestingly, the transgenics also showed enhanced endogenous cytokinin level as compared to WT, which, further increased with salinity [39]. Similarly, the over expression of potato PR-10a (formerly STH-2) gene significantly enhanced salt and osmotic stress tolerance in transgenic potato suspension cultures [46]. Constitutive expression of pea PR-10a showed enhanced seed germination of Brassica napus under saline conditions [47]. Recently, Jain et al. [48], showed that overexpression of AhSSIPR10 gene from peanut enhanced abiostic stress tolerance by reducing ionic and oxidative stress in transgenic plants exposed to salinity, heavy metal or drought stress.

The photosynthesis efficacy of WT and transgenics was analyzed by gradual NaCl stress, as Shavrukov [49] recommend that imposition of gradual salinity treatments reflects the natural condition in which salinity exists in ecosystems. The wild type tobacco plants under salinity showed noticeably lower photosynthesis, stomatal conductance, and maximum quantum yield of CO2 assimilation, intercellular CO2 concentration, ratio of intercellular to ambient CO2 concentration, stomatal limitation value and non-photochemical quenching as compared to control plants. On the other hand, transgenic plants under salinity showed lower transpiration, higher photosynthetic parameters and WUE [39]. The overexpression of the AhSSIPR10 gene in tobacco showed increased photosynthesis during abiotic stress [48]. The enhanced endogenous cytokinin level of transgenics under control as well as stressed condition might also be protecting the photosynthetic machinery. Interestingly, Cortleven and Valcke [50] reported that both an increase as well as a decrease in cytokinin content results in a better photosynthetic performance, as cytokinins can induce changes in the kinetics of the electron transfer reactions in PSII during photosynthesis.

**JcPR-10a transgenics exhibit tolerance against Macrophomina phaseolina fungus:** The in vitro analyses revealed that JcPR-10a has both RNase and antifungal activity, whereas boiled protein lacked RNase and antimicrobial activity [32]. The fungal resistance assays of JcPR-10a transgenics elucidated the potential role of JcPR-10a in enhancing resistance against Macrophomina. In the leaf assay, 3 dpi of Macrophomina showed severe blackening along the mid vein and side veins from proximal to distal end of the WT leaf only. Furthermore, in the leaf extract assay the radial growth and intensity of microsclerotia is greatly inhibited with the transgenic leaf extract, indicating that the JcPR-10a protein shows antifungal activity. This could be due to induced RNase activity. The JcPR-10a transgenics did not show enhanced SA concentrations under normal growth conditions but it might get
might be working in co-

JcPR-10a functions. It can be speculated that maintaining cellular redox potential with reduced oxidative damage development and photosynthesis by functioning as antioxidants, exposed to salinity stress, the increased cytokinin promote growth, endogenous cytokinin levels in planta, as a result when the plants are normal growth and development.

Concluding Remarks

Mechanism of JcPR-10a regulation

The constitutive overexpression of JcPR-10a protein, enhances the endogenous cytokinin levels in planta, as a result when the plants are exposed to salinity stress, the increased cytokinin promote growth, development and photosynthesis by functioning as antioxidants, maintaining cellular redox potential with reduced oxidative damage and enhanced water use efficiency during salinity.

However, during implication of biotic stress on Macrophomina infection, it can be postulated that SA content increases as infection leads to accumulation of SA (Figure 1). Argueso et al. [51] reported a possible feedback loop of SA on cytokinin signaling that would work to fine-tune the level of defense responses to pathogens, and that cytokinin levels are important for plant immunity. According to Zubini et al. [33] a correlation exists between RNase hydrolysis and cytokinin binding; suggesting a mechanism of alternate zeatin/RNA binding to PR-10 protein. Therefore with increasing SA, the cytokinin decreases, facilitating binding of RNA to JcPR-10a protein causing increased RNase activity leading to programmed cell death around the infection sites. Whereas, under normal conditions, higher binding of cytokinin to JcPR-10a protein results in reduced or no RNase activity, facilitating normal growth and development.

Concluding Remarks

JcPR-10a represents an important pathogenesis related gene that potentially exploits its cytokinin binding affinity in performing diverse functions. It can be speculated that JcPR-10a might be working in co-ordination with cytokinin signaling in mitigating the stress induced damage by regulating different stress signaling pathways, leading to enhanced stress tolerance. The RNase activity of PR-10 proteins regarded to be directly or indirectly involved with antifungal role also might get modulated by phosphorylation. Post translational modification especially phosphorylation might be involved in regulating the function of PR-10 genes, as reported for CaPR-10a protein [52]. JcPR-10a protein showed the presence of phosphorylation sites, however, further studies need to be carried out. Also the role of JcPR-10a protein towards antiviral activity is being explored. JcPR-10a gene can serve as an important candidate gene to overcome the disease problem in Jatropha by enhancing the defence potential of Jatropha, an important biofuel crop, being greatly advocated for growing in wastelands.

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